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Comparative Field Evaluation of the Fluorescent-Antibody Test, Virus Isolation from Tissue Culture, and Enzyme Immunodiagnosis for Rapid Laboratory Diagnosis of Rabies

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The rabies tissue culture infection test (RTCIT) and rapid rabies enzyme immunodiagnosis (RREID) were compared to the fluorescent-antibody test (FAT) with field specimens. At the French National Reference Center for Rabies, 15,248 specimens were analyzed by FAT and RTCIT, and 2,290 of those specimens were also tested by RREID; 818 other specimens were tested by FAT and RREID in 12 laboratories located in Africa, Asia, and Latin America. The sensitivities and specificities of RREID and RTCIT were comparable. This study showed that both tests can be used as backup procedures to confirm FAT. RREID is also strongly recommended for epidemiological studies and for laboratories which are not equipped for performing FAT.

The prophylaxis of human rabies is a constant problem, especially in developing countries (14). Estimates of the annual incidence of human rabies cases worldwide exceed 25,000. Since the disease in humans can be prevented by timely postexposure treatment, rapid and sensitive tests for the routine diagnosis of rabies in biting animals are required.

In 1903, Negri (9) described cytoplasmic inclusions in the brains of rabid animals. The presence of these inclusions had been the major diagnostic criterion up to 1958, when Goldwasser et al. (6) and later Dean and Abelseth (2) developed the fluorescent-antibody test (FAT) for rabies diagnosis. FAT has become the recommended procedure because it is fast, inexpensive, and reliable when performed in a competent laboratory with high-quality reagents. Nevertheless, the importance of the laboratory diagnosis for a medical decision prompted the use of virus isolation as a routine backup procedure. Virus isolation permits the unambiguous identification of the etiologic agent, as well as its preservation for further studies. The mouse inoculation test (MIT) developed by Webster and Dawson (18), still in use in some countries, was first carried out. But MIT yields delayed results: the usual incubation period in adult mice is between 7 and 20 days. This delay can be shortened to 5 or 7 days by performing MIT in newborn mice, but a positive result many days after a human exposure is of limited value. It has been replaced at the French National Reference Center for Rabies since 1982 with the rabies tissue culture infection test (RTCIT) employing murine neuroblastoma cells (N2a) (16). RTCIT provides results within a time when postexposure immunization is still considered efficacious, but it requires technical expertise and is quite expensive. Nevertheless, FAT and RTCIT remain the standards of rabies diagnosis against which newly developed diagnostic approaches including dot hybridization (4) and enzyme immunoassays should be compared. What was clearly needed was a sensitive and inexpensive technique having an objective cutoff point which could be easily applied to large numbers of specimens. With these goals in mind, a new method based on the immunocapture of rabies antigen (enzyme-linked immunosorbent assay sandwich technique) called rapid rabies enzyme immunodiagnosis (RREID) was recently developed (10). Preliminary results showed that RREID was a useful tool for the diagnosis of rabies in epidemiological studies and as a backup procedure for FAT (11). The incubation of a positive specimen in the wells of the sensitized microplate results in the binding of rabies nucleocapsid to the antinucleocapsid antibody bound to the solid phase. The bound viral antigen is then quantified with the same antinucleocapsid antibody conjugated to peroxidase; the color appears when the substrate is added.

In the present study, our purpose was to assess by a field study the respective qualities and drawbacks of FAT, RTCIT, and RREID. We compared the results obtained by FAT and RTCIT on 15,248 field specimens and by FAT, RTCIT, and RREID on 2,290 of these specimens. All these specimens, received at the French National Reference Center for Rabies, were from animals considered to be responsible for human exposure. This study reports also the results of FAT and RREID obtained on 818 field specimens in 12 laboratories in Africa, Latin America, and Asia. These results and the properties of each diagnostic test are discussed.

MATERIALS AND METHODS

FAT. In the French National Reference Center for Rabies, FAT was performed on impression smears of Ammon's horn and brain stem (no smears of cortex) fixed for 30 min in cold acetone by the technique described by Dean and Abelseth (2). Staining was accomplished by covering the slides for 30 min at 37°C with rabies antinucleocapsid rabbit immunoglobulin G conjugated with fluorescein isothiocyanate and adsorbed with 10% normal mouse brain tissue (Diagnostics Pasteur, Marnes la Coquette, France). Evans blue (1/5,000) was used as a counterstain. The slides were washed by immersion in phosphate-buffered saline (pH 7.6) for 5 min. A mounting glycerine medium was used, and the slides were examined under cover slips at ×400 by using an epifluorescence microscope equipped with an ultra-high-pressure mercury UV lamp. All slides were read by two experienced microscopists performing daily rabies diagnosis. On the positive slides, intracytoplasmic viral antigen could be observed as minute granules or oval shaped inclusions scattered in the pericaryon.

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For the collaborating field laboratories, no details are available on the conjugate and microscopy equipment used.

RTCIT. RTCIT was performed according to a modification of the technique described by Portnoi (D. Portnoi, S. Favre, and P. Sureau, Rabies Inform. Exch., Centers for Disease Control **6**:35–36, 1982).

Small pieces of brain stem, cortex, and Ammon's horn of each specimen were homogenized and prepared as a 1:3 (vol/vol) suspension in Dulbecco modified Eagle medium adjusted to pH 7 with $Na(HCO_3)_2$ and supplemented with 40% calf serum, vancomycin (0.4 mg/ml), gentamycin (30 μg/ml), and amphotericin B (40 μg/ml). The suspension was clarified by centrifugation at 1,500 × *g* for 30 min at 4°C.

Each well of eight-chamber slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) was seeded with 0.4 ml of Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and containing a suspension of 4×10^5 viable murine neuroblastoma cells (ATCC CCL 131) per ml. Supernatants (50 μl) of each specimen to be examined were added to individual wells and mixed with the cell suspension by gentle agitation. After 18 h of incubation at 37°C in a humidified incubator with a 5% CO_2 atmosphere, the supernatant medium was carefully aspirated. The chamber frames were then removed, the slides were air dried, and the N2a monolayer was fixed in cold acetone for 30 min and stained with the rabies antinucleocapsid conjugate.

RREID. The preparation of the specimen supernatants followed the same steps as those for RTCIT. The reaction was performed by the procedure described by Perrin and others (10, 11). The clarified supernatants of each specimen were distributed in duplicate in the wells of the sensitized microplates (200 μl per well). The plates were then incubated for 1 h at 37°C. After repeated washings with phosphate-buffered saline–Tween, each well received 200 μl of antirabies nucleocapsid rabbit immunoglobulin G conjugated with horseradish peroxidase. The plates were incubated for 1 h at 37°C and then washed again. Finally, the chromogen-substrate mixture (*ortho*-phenylenediamine and hydrogen peroxide) was added (200 μl per well). The plates were left for 20 min at room temperature to allow for color development, which was stopped by adding 4 N H_2SO_4 (50 μl per well). The color was then evaluated qualitatively with the naked eye, and the absorbance was measured quantitatively by using a Titertek Multiskan spectrophotometer (Flow Laboratories S.A., Puteaux, France). The A_{492} was compared with those of a positive and a negative control. According to the recommendations of the manufacturer, samples whose absorbance was greater than 0.08 absorbance units above that of the negative control were considered positive. All the reagents of RREID are now commercially available (Diagnostics Pasteur).

Origin of specimens. (i) French National Reference Center for Rabies (Institut Pasteur, Paris). Specimens (n = 15,248) from more than 40 animal species received from June 1984 to August 1988 were analyzed by FAT and RTCIT. Of these specimens, 2,290 were also analyzed by RREID.

(ii) Field laboratories. Twelve other laboratories located in Africa, Latin America, and Asia (listed in Acknowledgments) participated in an evaluation of RREID in field conditions. Specimens (n = 818) from 19 different animal species were examined following the instructions supplied with the kits. These results were compared with those obtained by FAT.

RESULTS

Results obtained at the French National Reference Center for Rabies. The results obtained on 15,248 specimens are summarized in Table 1. Of the 1,511 specimens positive by FAT, 19 (1.25%) were negative by RTCIT. No specimen was negative by FAT and positive by RTCIT. The concordance was 99.75%. These results were analyzed according to the animal species. They were representative of the number of specimens analyzed from each species. It was noted that the 19 specimens that gave the discordant results were all putrid because of the long delay of conservation before examination, which was probably responsible for the virus inactivation resulting in the negative result of the RTCIT.

TABLE 1. Correlation between the results obtained by FAT and RTCIT on 15,248 field specimens received at the French National Reference Center for Rabies

RTCIT result	No. of FAT results		
	+	−	Total
+	1,492	0	1,492
−	19	13,737	13,756
Total	1,511	13,737	15,248

The results obtained on 2,290 specimens by FAT, RTCIT, and RREID are presented in Table 2. There was almost no difference between the results obtained after reading with the naked eye and after analysis of the spectrophotometer data (Table 3). Only one specimen positive by FAT and negative by RREID by spectrophotometer analysis was found positive by RREID after naked-eye reading. Three false-positive results by RREID were found either after naked-eye reading or after spectrophotometer analysis. We further considered only the absorbance data collected by spectrophotometry. Of the 302 specimens that were positive by FAT, 5 (1.7%) also positive by RTCIT were negative by RREID, 7 (2.3%) were negative by RTCIT but positive by RREID, and 10 (3.3%) were negative by both RTCIT and RREID. Of the 1,988 specimens that were negative by FAT and RTCIT, only 3 (1.5%) were positive by RREID. The concordance of FAT with RTCIT was 99.26% and of FAT with RREID was 99.21%. One specimen not presented in Table 2 was too putrid to give suitable results by FAT and RTCIT but showed a positive reaction by RREID. No correlation can be found between animal species and discordant results within the different tests (Table 4).

Postulating that the reference test (FAT) had a specificity and a sensitivity of 100%, we calculated the specificities and sensitivities of RTCIT and RREID (Table 5). RTCIT showed a higher specificity (100%) than RREID (99.85%), but its sensitivity (94.37%), possibly explained by virus inactivation in some specimens, was lower than that of RREID (95.03%).

The sample of 2,290 specimens was not representative of the 15,248 specimens that we have received since 1984. As we began to evaluate the RREID technique, we preferentially tested specimens giving positive or doubtful or discor-

TABLE 2. Correlation between the results obtained by FAT, RTCIT, and RREID on 2,290 field specimens received at the French National Reference Center for Rabies

FAT result	No. of results				Total
	RTCIT+ RREID+	RTCIT+ RREID−	RTCIT− RREID+	RTCIT− RREID−	
+	280	5	7	10	302
−	0	0	3	1,985	1,988

TABLE 3. Correlation between the results in RREID after naked-eye reading (EYE) and after analysis of the spectrophotometer data (SPEC)

FAT result	No. of results				
	SPEC+ EYE+	SPEC+ EYE−	SPEC− EYE+	SPEC− EYE−	Total
+	287	0	1	14	302
−	0	3	3	1,982	1,988

dant results by FAT and RTCIT. This explains why the prevalence was higher and the sensitivities were lower in the sample of 2,290 specimens than in the 15,248 specimens.

When specimens were examined in duplicate by RREID, no discordance was observed between the duplicate optical density (OD) values. The coefficient of variation (the measurement of variability of results in replicate wells) estimated on 300 samples was 2.27%. The distribution of the quantitative OD values obtained by RREID with respect to the qualitative results obtained by FAT is shown in Table 6. We deduced from the negative control mean OD value of 0.041 (standard error of the mean = 0.019) the cutoff mean OD value of 0.121 ± 0.019. This value separates OD results of specimens regarded as negative from those regarded as positive. The discriminating power of RREID is illustrated in Table 6. More than 92% of the negative specimens had an absorbance lower than 0.050 OD units and more than 86% of the positive specimens had an absorbance higher than 0.400 OD units. Nevertheless, 3% of the positive samples had a low OD value (<0.050).

Results obtained in 12 field laboratories. The results obtained on 818 specimens are presented in Table 7. Four hundred twenty-eight results were positive and 363 were negative by both FAT and RREID. Five were negative by FAT and positive by RREID, and 22 were positive by FAT and negative by RREID. RREID showed a high concordance with FAT. The specificity and the sensitivity were 98.64 and 95.11%, respectively. This specificity is lower than that obtained at the French National Reference Center for Rabies. No information was given about the reasons for the discordant results. Since these laboratories were performing the RREID for the first time, one may expect an improvement of their results when they become more familiar with this new technique.

The predictive value (PV) model (3) can be used in rabies diagnosis to evaluate whether a positive or a negative result is correct. The predictive value of a positive result (PV+) is the percentage of true-positive results with respect to all specimens with positive results. Similarly, the predictive value of a negative result (PV−) is the percentage of true-negative results in all specimens which have negative results. These values (Table 5) give the probability of rabies infection from the sensitivity and specificity of the test and from the prevalence estimated. RTCIT had a higher PV+ than RREID (100 and 98.96%, respectively). Conversely, RREID had a PV− slightly higher than that of RTCIT (99.25 and 99.15%, respectively). Indices of "gain in certainty" which depend directly on the sum of the specificity and sensitivity were found to express more comprehensively the performance of a diagnostic test (1). We used the term gain in certainty to describe the change in the FAT diagnosis estimate of a specimen of being rabid or not rabid that occurs as a result of RTCIT or RREID testing. The expected net gain defines test performance by combining gain in certainty with the likelihood that gain will occur in practice. The expected percent gains in certainty [E(%C)] are shown in Table 5. The E(%C) measure of RREID ranged from 93.19 to 94.88%, according to the different laboratory origin of the results. The E(%C) measures of RTCIT were 94.37 and 98.74%, according to the number of specimens considered.

TABLE 4. Animal species in relation to discordant results by FAT, RTCIT, and RREID on 2,290 field specimens received at the French National Reference Center for Rabies

Animal species	No. of FAT results			
	Positive			Negative (RTCIT−, RREID+)
	RTCIT+ RREID−	RTCIT− RREID+	RTCIT− RREID−	
Fox	1	5	2	0
Dog	2	0	1	2
Cat	1	0	1	1
Cattle	0	2	1	0
Sheep	1	0	3	0
Horse	0	0	1	0
Roe deer	0	0	1	0

DISCUSSION

FAT is the most widely used procedure for rapid rabies laboratory diagnosis. In 1975, Kissling (8) evaluated that FAT was identifying at least 98% of rabies-infected brain tissue submitted to diagnosis, with a total agreement of more than 99% with the MIT. The constant improvement of the technique, especially the introduction of epifluorescence equipment, and the improved quality of the antirabies conjugate increased its accuracy significantly. More recent publications on laboratory technique comparisons (5, 11, 14), as well as our experience, support the opinion that FAT is the standard for rabies diagnosis against which the other diagnostic approaches should be compared. For the purpose of this study, we postulated that there was no more precise estimation of the prevalence of rabies in the specimens submitted for diagnosis than the proportion of positive FAT results. This means that we considered specificity and sensitivity of FAT to be 100%. FAT was also found to be fast and inexpensive. Nevertheless, it has two major drawbacks. It cannot be applied with ease to a large number of samples, and some laboratories in developing countries cannot afford the cost of the fluorescence microscope equipment and cannot, above all, ensure its maintenance.

Both RTCIT and RREID results showed a high concordance with FAT in our hands. It was due to a large part to their specificities, which were greater than 99.85%. Their sensitivities were slightly lower (around 95%), corroborating that the use of these tests instead of FAT is not recommended (11).

The result obtained by 12 laboratories in developing countries clearly confirmed that, in field conditions, the sensitivity and reliability of RREID are practically not decreased. These results are quite similar to those obtained by five W.H.O. and national reference centers for rabies (Federal Republic of Germany, Canada, and the United States) (11) (Table 5).

Assuming that sensitivity and specificity are intrinsic properties of a diagnostic test relative to detecting infection, we then tried to measure how strongly RTCIT and RREID argue for the presence or absence of rabies given these intrinsic properties. There has been little consensus about

TABLE 5. Intrinsic and extrinsic properties of RTCIT and RREID in different epidemiological situations[a]

No. of specimens	Origin	%												
		Concordance		Specificity		Sensitivity		Prevalence	PV+		PV−		Combined Expected Gain in Certainty	
		FAT RTCIT	FAT RREID	RTCIT	RREID	RTCIT	RREID		RTCIT	RREID	RTCIT	RREID	RTCIT	RREID
15,248	France	99.75		100		98.74		9.91	100		99.86		98.74	
2,290	France	99.26	99.21	100	99.85	94.37	95.03	13.19	100	98.96	99.15	99.25	94.37	94.88
818	12 Laboratories (Africa, Asia, Central and South America)		96.69		98.64		95.11	52.93		98.85		94.28		93.75
779[b]	5 Laboratories (Europe and North America)		96.67		96.44		96.75	71.12		98.53		92.34		93.19

[a] TP, True-positive; TN, true-negative; FP, false-positive; FN, false-negative; sensitivity = TP/(TP + FN); specificity = TN/(TN + FP); PV+ = TP/(TP + FP); PV− = TN/(TN + FN); combined expected gain in certainty = sensitivity + specificity −1.
[b] Published in reference 11.

the problem of evaluating this aspect of test performance. Nevertheless, we chose two indicators: the PV model (3) and the measure of gain in certainty (1).

The PV model is very influenced by prevalence (3). As the prevalence of rabies increases in the specimens submitted for diagnosis, the PV+ increases and, conversely, the PV− decreases. This can easily be seen in Table 5. To answer the question of how high these values should be, we must consider the justification for antirabies treatment. Considering that there is no more risk of postvaccinal reactions with the new purified cell culture rabies vaccines, we should keep in mind only how high the PV− is. The PV− of RREID, when it is practiced daily, is very good (99.25%) and slightly higher than that of RTCIT.

The second indicator is the measure of the combined expected net gain in certainty (1). An expected gain of 0 means that the test gives no information, while the maximum of information is obtained when the value reaches 100. The E(%C) values are high but lower than 100%. This means that RTCIT and RREID give a little bit less information than FAT. It also reflects that specificity and sensitivity are not 100% but are not far from this value.

Several cell lines, such as murine neuroblastoma cells (N2a) (7), CER (15), and BHK-21 (13), have been shown to be suitable for primary isolation of rabies virus from field specimens. We used N2a because its superior sensitivity over other other cell lines and over MIT was previously demonstrated (12, 13, 15, 17). In our opinion, this sensitivity obviates the need for sensitization of the cell with DEAE dextran. We would also mention that these cells can be stored in suspension at 4°C in a quiescent state for at least 3 days. This provides an easy access to cells for daily viral isolation. Furthermore, in our daily practice, RTCIT gives results within 18 h. Reports from other laboratories recommend allowing the cells to grow for 3 or 5 days before giving the result (B. Roseneau, R. Davenport, and K. Girard, Rabies Inform. Exch., Centers for Disease Control **14**:1–5, 1986; R. J. Rudd and C. V. Trimarchi, Rabies Inform. Exch., Centers for Disease Control **14**:33–35, 1986). Our data, corroborating the previous studies (B. Roseneau, R. Davenport, and K. Girard, Rabies Inform. Exch., Centers for Disease Control **15**:5–9, 1986) showed that positive findings are obtained most likely within 1 day. This means that in field conditions the eventual increased sensitivity obtained by incubation periods longer than 18 h would not be worth the additional delay in obtaining the diagnosis.

Nevertheless, RREID is still faster than RTCIT. A laboratory performing FAT or RREID or both is able to give positive results within one day. There is no need for testing specimens in duplicate, and RREID is also easily automated. Furthermore, the threshold level, obtained by adding 0.08 absorbance units to the OD of the negative control, gives an optimum discrimination between positive and negative cases. Of the specimens shown positive by FAT and RTCIT, 1.7% were missed by RREID. This indicates that this test should not replace FAT where FAT is presently performed. These findings corroborate the previous studies on RREID (10, 11). Further studies will be undertaken to explain why about 3.3% of the specimens positive by FAT are found negative by RTCIT and RREID. At this stage, we cannot tell if these specimens were really rabid or gave false-positive results by FAT, because there is no technique more sensitive than these three actually available. We should add only that these specimens gave weak reactions by FAT. One other major characteristic of RREID is that its reliability is not affected by the putrid condition that some specimens may present. Furthermore, it can be performed on inactivated specimens (2 h at 56°C). The purified antinucleocapsid rabbit

TABLE 6. Distribution of OD values obtained by RREID according to the results found by FAT

FAT result	No. of RREID results (% of total no.)						
	0–0.050	0.050–0.100	0.100–0.150	0.150–0.200	0.200–0.400	0.400–2.000	Total
−	1,846 (92.86)	134 (6.74)	6 (0.30)	2 (0.10)	0 (0)	0 (0)	1,988
+	11 (3.64)	3 (0.99)	1 (0.33)	6 (1.99)	19 (6.29)	262 (86.76)	302

TABLE 7. Results from 12 laboratories which compared FAT and RREID[a]

Laboratory	Total no. of samples	No. of RREID results			
		Positive		Negative	
		FAT+	FAT−	FAT+	FAT−
A	93	73	0	3	17
B	94	37	0	3	54
C	50	28	0	0	22
D1	68	47	0	11	10
D2	63	31	0	1	31
E	78	23	1	1	53
F	87	17	0	0	70
G	42	28	0	0	14
H	34	13	0	0	21
I	30	9	0	1	20
J	12	8	1	1	2
K	64	27	0	1	36
L	103	87	3	0	13
Total	818	428	5	22	363

[a] Laboratories are given in Acknowledgments.

immunoglobulin G used in RREID is prepared with the Pasteur strain of rabies virus (PV-PARIS/BHK). The test is then recommended for diagnosis of lyssavirus of serotype 1. However, other serotypes of lyssavirus (Lagos Bat, Mokola, and Duvenhage) can be detected (11).

This study clearly showed that RTCIT and RREID results are quite comparable even in field laboratory conditions, but the sensitivity of RREID is slightly higher than that of RTCIT. RTCIT and RREID could be very useful to many laboratories that now rely exclusively on FAT results or still use MIT as a back up procedure to corroborate FAT results. However, such laboratories should keep in mind that RTCIT requires more costly equipment than RREID, as well as workers trained in cell culture; the cost per test of both tests is the same (J. Barrat, H. Bourhy, and J. H. Cox, Le diagnostic de la rage et le typage des virus rabiques. I. Le diagnostic de la rage. Proc. 2nd W.H.O. Meet. Control of Rabies in Europe, Annecy, 8 to 10 June 1988, in press). On the other hand, RREID can be strongly recommended for epizootiological surveys and for laboratories which are not equipped for performing FAT.

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